The Effect of Dark Adaptation on Red and Blue Light-Driven Pupil Responses

Thesis

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Abstract

Purpose: Time spent outdoors has been consistently associated with delaying the onset of myopia. This association may be due to the involvement of intrinsically photosensitive retinal ganglion cells (ipRGCs), considering their role in long-term ambient illumination detection and connection with dopaminergic amacrine cells. Pupil responses driven by ipRGC input have typically been measured clinically in response to flashes of red and blue light following dark adaptation. However, this is not a feasible method for testing children, as dark adaptation adds a significant amount of time to each trial. The purpose of this study was to evaluate the effect of dark adaptation on the pupil responses to red and blue light stimulation.

Methods: Subjects were 20 adults age 24.0 ± 2.37 years (average ± SD), 55% female, with an average spherical equivalent (SEQ) refractive error of -3.22 ± 2.78 diopters (Grand Seiko WR-5100K cycloplegic autorefraction), ranging from -10.61 to +0.77 diopters. The RAPDx pupilometer measured pupil sizes in response to blue and/or red light oscillating at a low temporal frequency of 0.1Hz. The standard testing protocol included 5 minutes of dark adaptation prior to each of three stimulus conditions: alternating red and blue, red-only, then blue-only. Subjects repeated this standard protocol without dark adaptation. This study also evaluated whether pupil responses might be enhanced by two minutes of red-only stimulation (instead of the standard one

minute), once with and once without dark adaptation. These 4 test conditions were conducted in random order on separate days. The primary outcome measure was the difference in normalized pupil size in response to blue-only light compared to blue during the alternating condition.

Results: Consistent with previous research, pupils tended to become more constricted with repeated exposure to blue light. The average difference (±SD) in normalized pupil size between blue-only and alternating blue conditions with dark adaptation was 10 ± 2.4%. Without dark adaptation, the pupil did not become as constricted with repeated exposure to blue light, with the difference reduced by 2.8% (repeated measures ANOVA; $p<0.0001$). Compared to the standard protocol, the longer exposure to red light had no significant effect on the pupil responses to repeated pulses of blue light (0.8% difference, $p = 0.18$).

Conclusion: Dark adaptation resulted in a significant reduction in pupil size in response to repeated pulses of blue light compared to the identical protocols without dark adaptation. Increasing the exposure to red light had no effect on pupil size in response to repeated pulses of blue light. Use of dark adaptation needs to be taken into account during pupillary testing. This effect could be attributed to an increase in the amount of time between light exposures allowing dopamine to diffuse throughout the retina and decrease the threshold levels of the retinal cells driving the pupil response.

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Chapter 1. Introduction

Myopia, or nearsightedness, is a condition characterized by an eye with too much convergent refractive power for the physical length of the eye. This mismatch between focal length and physical length is commonly due to steeper corneal curvatures, more powerful crystalline lenses, increased axial length, or a combination of these factors. This mismatch leads to the creation of an image that is focused in front of the retina, resulting in symptoms of optical blur when looking at distant objects. The estimated prevalence of any degree of myopia among people aged 12-54 years of age has been estimated to be around 25% (Sperduto, Seigel et al., 1983). However, more recent studies have shown an increase in the prevalence of myopia, particularly in the Asian population. In a study by Vitale et al., researchers estimated the prevalence of myopia among people aged 12-54 years of age to be around 41.6% (Vitale, Sperduto et al., 2009). In a previous study by Vitale et al., the prevalence of myopia in the United States had been estimated to be 33.1% (Vitale, Ellwein et al., 2008). It is apparent from these two studies that the method of estimating prevalence can change the outcome. Regardless of the difference in estimation, the jump in prevalence from 25% to 33.1%, or to 41.6%, over the time span between these studies is a particularly troubling statistic. In addition to increasing prevalence, another alarming trend is that the onset of myopia appears to be occurring at younger ages. In one large-scale, cross-sectional survey of children ages 7 to 18,

researchers found that the mean refractive error tended to be myopic at progressively younger ages over the years in which the study was conducted. In 1983, the mean refractive error became myopic in school aged children by age 11. However, in 2000, the mean refractive error became myopic in children by the time they were 8 years old (Lin, Shih et al., 2004). Further, there have been studies suggesting that the increasing prevalence of myopia will not slow down any time soon. In a meta-analysis, Holden et al. predicted that by the year 2050 myopia will affect 5 billion people, and high myopia over -5D will affect 1 billion people (Holden, Fricke et al., 2016).

The increasing prevalence and the earlier age of onset of myopia are troubling for many reasons. Myopia currently does not have a cure, although there are ways in which the optical image can be improved. Corrective diverging lenses in the form of spectacles or contact lenses can be utilized in order to focus light onto the retina, forming a clear image for the brain to interpret. Advances in refractive surgeries have also made options such as LASIK or PRK available for correcting myopia. Additionally, procedures such as cataract surgery or clear lens extraction may also be utilized for the correction of high myopia. The myopia epidemic is something that warrants significant attention and research, as it leads to financial burden and increases threats to vision. The financial burden caused by uncorrected distance refractive error was estimated to be $202 billion per year, largely due to myopia (Fricke, Holden et al., 2012).

In addition to financial burden, there are numerous increased threats to vision due to myopia. These threats are usually associated with the excessive axial length that is often

found in high myopes. High myopia increases the risk of many pathological changes such as glaucoma, retinal detachments, many forms of cataracts, chorioretinal atrophy, and lacquer cracks (Saw, Gazzard et al., 2005). The increased risk of glaucoma in the presence of myopia remains even in the absence of other glaucoma risk factors and regardless of IOP measurements. A study conducted by Mitchell et al. concluded that myopic subjects had a twofold to threefold increased risk of glaucoma when compared to nonmyopic subjects. This increased risk remained even after adjusting for known glaucoma risk factors, such as increased IOP (Mitchell, Hourihan et al., 1999). The increased threat to vision alone provides a compelling reason to work towards solving the myopia epidemic, as these pathological changes can all lead to irreversible vision loss that cannot be corrected by refractive means.

There are certain known risk factors in the development of myopia. One of these risk factors is genetics. One study supporting the influence of genetics in the development of myopia analyzed the refractive errors of monozygotic and dizygotic twins. This study found that the correlation of spherical equivalent between monozygotic twins was almost twice as high when compared to the correlation for dizygotic twins, suggesting a strong genetic effect. This genetic effect was determined to play a major role in the development of many refractive states, including myopia, hyperopia, and astigmatism (Hammond, Snieder et al., 2001). In another study involving school-aged children, the number of myopic parents was determined to be predictive in whether or not the child would also become myopic. Parental history of myopia was also found to influence the effect of myopia development when analyzed with the number of hours the child spent outdoors.

Children with two myopic parents and lower amounts of outdoor activity had an increased chance of becoming myopic. This increased chance was greater for those children with two myopic parents when compared to those children with one or no myopic parents (Jones, Sinnott et al., 2007). In a genome-wide association meta-analysis, Tedja et al. identified 161 independent susceptibility loci related to the development of refractive error. These identified loci are thought to contribute to pathways involved in axial elongation, specifically "a retina-to-sclera signaling cascade that induces scleral remodeling in response to light stimuli" (Tedja, Wojciechowski et al., 2018).

It is clear from the literature that there is a genetic component to refractive error development. However, it has also been shown that genetics is not the only factor in the development of refractive error. A large amount of research has also been conducted that suggests refractive error is influenced by a combination of genetics and environmental factors related to lifestyle. Some of the widely researched lifestyle risk factors include near work and time spent outdoors. The influence of near work in the development of myopia has been a highly researched as well as a highly debated topic. In a study by Mutti et al., researchers found that both heredity and near work were significantly associated with children being myopic, although heredity seemed to have a stronger role. The study recognized that there could be an intimate association between near work, parental myopia, and a child's myopia in that it is possible that myopic parents pass on a myopigenic environment involving high near work demands, perpetuating a familial cycle of myopia. However, upon addressing this potentially confounding association, the study found no evidence to support this theory that heredity is only important because

myopic parents encourage an environment of more near work. Odds ratios for a child being myopic associated with parental myopia were unaffected by adjusting for near work (Mutti, Mitchell et al., 2002). Another study conducted by Saw et al. found that although not all quantitative measures of near work were associated with myopia, the number of books read per week was positively associated with myopia. They also found that books read per week interacted with parental myopia, suggesting that the effects of near work might be worse when there is parental myopia (Saw, Chua et al., 2002).

The previously mentioned studies established evidence supporting the idea that myopia is associated with increased near work. However, these studies did not address the question as to whether or not performing more near work tasks would increase the chance of developing myopia. A study completed by Jones et al. examined survey-based data from the Orinda Longitudinal Study of Myopia in an attempt to identify risk factors for developing myopia. Variables were analyzed for third graders who would become myopic versus those who would not. Reading hours per week was determined not to be a statistically significant factor associated with the development of myopia after controlling for sports and outdoor hours per week (Jones et al., 2007). In another longitudinal study on Australian schoolchildren, French et al. found that parental myopia and ethnicity were associated with the development of myopia, while near work was only significant in the younger subject cohort in the study. The younger cohort consisted of children who were six years old, as opposed to the older cohort consisting of children who were twelve years old. (French, Morgan et al., 2013). In a study examining risk factors for the development of myopia in Singaporean children, Saw et al. similarly found evidence suggesting a link

between parental myopia and the development of myopia in their children. This study also suggested a link between Intelligence Quotient (IQ) scores and the risk of developing myopia. However, this study much like the other two studies found that reading in books per week was not associated with the development of myopia (Saw, Shankar et al., 2006). Although myopes have been found to perform increased amounts of near work when examined in cross-sectional studies, these longitudinal studies indicate that near work does not increase the risk for the development of myopia.

It seems logical then to ask if near work does not increase the risk of onset of myopia, does it speed the progression of myopia in those children who have already been diagnosed? A study conducted by Jones-Jordan et al. analyzed data from the Collaborative Longitudinal Evaluation of Ethnicity and Refractive Error (CLEERE) Study to determine which factors, if any, were associated with the progression of myopia. The researchers found that near work had little effect on the rate of myopia progression (Jones-Jordan, Sinnott et al., 2012).

The amount of time emmetropic children spend outdoors may be a more important environmental variable than near work. Cross sectional studies suggest that children who are myopic spend less time outdoors compared to their non-myopic counterparts. Even after adjusting for near work, parental myopia, and ethnicity, Rose et al. found higher levels of total time spent outdoors were associated with more hyperopic/less myopic mean refractions in twelve-year-old students. The tendency towards a relatively more hyperopic refraction was found in children who spent more time outdoors, regardless of

the activity. The result was the same for children playing sports, participating in picnics, or simply walking. Time spent on indoor sports had no effect on the ultimate refractive error, pointing further to the idea that the time spent outdoors is the significant factor in the development of myopia, not the participation in sports activities (Rose, Morgan et al., 2008). In another study conducted by Dirani et al., researchers also found that the total outdoor activity time was significantly associated with myopia onset, and that the total time spent outdoors was associated with a significantly less myopic refraction and shorter axial lengths. They also found that the amount of near work was not predictive of outdoor activity time, and therefore concluded that outdoor activity should be considered to be an independent factor involved in the development of myopia (Dirani, Tong et al., 2009).

These cross-sectional studies suggest that children who are myopic spend less time outdoors, much like the studies suggesting that children who are myopic perform more near work. However, unlike the findings for near work, longitudinal studies show that time spent outdoors reduces the risk of the onset of myopia, suggesting a true protective effect. Jones et al. found that increased hours of participation in sports and other outdoor activities was associated with reduced odds of a child developing myopia. The analysis showed that differences were found between the children who would develop myopia compared to the group of children who would not for the number of myopic parents, the number of sports the child was involved in, and for hours spent outdoors per week. Lower amounts of sports and outdoor activity increased the odds that a child would become myopic (Jones et al., 2007). Another study conducted by French et al. again found that children who became myopic spent less time outdoors compared to children

who did not become myopic over the time of the study. The effect of time outdoors was seen in both the younger age group as well as the older age group of this study. Time spent outdoors, not near work, was the only variable significantly related to risk of myopia onset across both age groups (French et al., 2013). A clinical trial conducted in China randomizing children to increased time spent outdoors (one extra 40-minute period outdoors per school day) compared to their normal school schedule reduced the incidence rate of myopia. The three-year incidence rate was 30.4% in the group exposed to increased time spent outdoors, compared to 39.5% in the control group (p-value < 0.001) (He, Xiang et al., 2015).

Given the evidence to support that time spent outdoors likely plays a role in preventing or delaying the onset of myopia, the question becomes how does time spent outdoors play such a role. Although Rose et al. reported that the type of outdoor activity is not important regarding the onset of myopia, there have been studies suggesting that the physical activity performed while spending time outdoors could actually play a role in myopia development. Guggenheim et al. found that both time spent outdoors as well as physical activity were associated with a lower risk of incident myopia. However, the study also recognized that physical activity variables may have shown an association with incident myopia due to their inherent association with time spent outdoors. The authors concluded that time spent outdoors had a stronger correlation to myopia development than does physical activity (Guggenheim, Northstone et al., 2012).

With little evidence to support the independent role of exercise, another theory as to why time spent outdoors is preventative against the onset of myopia is the formation of vitamin D due to UVB exposure when outside. Mutti et al. investigated differences in circulating levels of vitamin D in young adults between myopes and non-myopes. After adjusting for differences in the intake of dietary vitamins, myopes were found to have lower average blood levels of vitamin D compared to non-myopes. However, the link between vitamin D and time spent outdoors was limited in this study, as time spent outdoors was not significantly related to myopia in the sample (Mutti and Marks, 2011). In a prospective study, Guggenheim et al. also sought to determine whether the underlying mechanism of the role of time outdoors in myopia development was associated with vitamin D. They found that total vitamin D and D3 levels were higher in those children who spent more time outdoors. Although time spent outdoors remained negatively associated with the development of myopia, levels of vitamin D were not associated with myopia development. Vitamin D may be a biomarker for time spent outdoors, however it appears that vitamin D is not related to the protective effects of time outdoors in myopia development (Guggenheim et al., 2012).

With little evidence for the role of physical activity and vitamin D in the protective effects seen from time outdoors, another aspect to be considered is the effect of visible light. One study, conducted by Ashby et al., examined the effect light exposure had on the development of refractive error and axial length in the chick animal model. In this study, chicks were fitted with translucent diffusers and were exposed to differing light levels for a period of time each day. The chicks were grouped according to differences in

light exposure. Ultimately the researchers found that the chicks exposed to high illuminances, sunlight, or intense laboratory lighting showed significantly shorter axial length as well as less myopic refractions when compared to those chicks exposed to normal laboratory lighting. These findings suggest that daily exposure to high illuminances may have a protective effect against the development of myopia. If these findings hold true in humans, this would point to light exposure playing a major role in the protective effect of time outdoors in the development of myopia (Ashby, Ohlendorf et al., 2009).

In another study conducted by Ashby and Schaeffel, researchers sought to identify why exposure to visible light could have such an effect on axial length. Researchers again found that high illuminance levels could slow the rate of myopia development when chicks were fitted with -7D lenses. The group exposed to high illuminance had a reduced rate of compensation for negative lenses when compared to the group of chicks exposed to normal laboratory illuminance. However, they also found that the level of light exposure did not change the set point for emmetropization, but rather delayed the development of the final level of induced refractive error. The effect of high illumination may be greater for form deprivation myopia than for lens-induced myopia (Smith, Hung et al., 2013). The key to understanding the effects of visible light became clearer in the second portion of their experiment. The researchers fit the chicks with diffusers, with one group raised under normal illuminance and a second under high illuminance. Researchers also injected the high illuminance group daily with either spiperone, a dopamine D2 antagonist, a vehicle solution composed of 0.1% ascorbic acid, or the chicks received no

injection, the last two serving as the control groups. They found that the protective effect of high illumination was essentially eliminated in the group receiving the intra-vitreal injections of spiperone, but the effect still held in the ascorbic acid group as well as the control group receiving no injection. This suggests that the effect of high illuminance in the development of myopia is perhaps mediated by the light-sensitive release of retinal dopamine (Ashby and Schaeffel, 2010).

In 2013, Feldkaemper and Schaeffer released a paper reviewing a wide body of literature supporting the hypothesis that dopamine is a retinal neurotransmitter that plays a key role in controlling eye growth. Reduced retinal dopamine levels have been observed in animal models deprived of a clear retinal image using either diffusers or negative lenses. Retinal dopamine levels were also observed to rise, and axial elongation was quickly inhibited upon removal of the diffusers or negative lenses. The effect of both diffusers and negative lenses in axial elongation can be mitigated with the addition of an intra-vitreal injection of a dopamine agonist. This again suggests that retinal dopamine is responsible for mediating the inhibitory signal for axial elongation. The authors note that pharmacological data has pointed to involvement of dopamine D2 receptors. These receptors are responsible for the coupling of rods, cones, and amacrine cell networks, ultimately leading to a spatial tuning of retinal neurons. This spatial tuning may be what determines the signaling for axial elongation. The majority of dopamine released throughout the retina comes from dopaminergic amacrine cells, and its release is stimulated through light exposure as well as retinal image contrast. Upon being released,

the dopamine spreads throughout the retina to reach dopamine receptors on the retina, choroid, and sclera (Feldkaemper and Schaeffel, 2013).

The light response of dopaminergic amacrine cells has been long understood to be driven by rod or cone photoreceptors through ON-bipolar cells. However, it has recently been found that input from these bipolar cells is not necessary to elicit a light-driven response from dopaminergic amacrine cells. This points to the idea that there is another retinal circuit involving the dopaminergic amacrine cells that is able to elicit excitatory light responses of these amacrine cells (Zhang, Wong et al., 2008). The driving force in this novel circuit has been identified as intrinsically photosensitive retinal ganglion cells (ipRGCs). These are photoreceptors within the retinal ganglion cell layer that express the photopigment melanopsin, which allows these ganglion cells to function as non-image forming photoreceptors. Research has shown that ipRGCs exhibit an excitatory influence on sustained dopaminergic amacrine neurons through the release of glutamate. Upon light stimulation, glutamate is released from the dendrites of the ipRGCs, and acts on the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors of the dopaminergic amacrine neurons. Dopaminergic amacrine neurons receive synaptic input from the ipRGCs in the inner plexiform layer of the retina, and they play a large role in changing the retinal response according to varying levels of illumination (Zhang et al., 2008). These M1-type ipRGCs in turn are also modulated through D1 dopamine receptors by the dopamine that is released. In a study conducted by Van Hook, Wong, and Berson, patch-clamp recordings of rat ipRGCs were obtained. These recordings showed that dopamine acts at the D1 dopamine receptors on the ipRGCs to alter the cell's

electrical membrane properties. Addition of a D1-family dopamine agonist attenuated the photocurrent recorded in the ipRGCs, causing a depolarization and reducing the input resistance of the ipRGCs. The effect was mediated through the stimulation of adenylate cyclase and the activation of Protein Kinase A (PKA). Based on these findings, adaptation of the responses from ipRGCs can be mediated by actions of the neuromodulator dopamine (Van Hook, Wong et al., 2012). Further evidence showing that ipRGCs are modulated by endogenous retinal neurochemicals was provided by Sodhi and Hartwick in a study examining the role of retinal adenosine levels in ipRGC responses. Retinal adenosine levels increase during prolonged periods of darkness, as opposed to dopamine levels which increase in periods of light stimulation. Sodhi and Hartwick showed that ipRGCs have A1 receptors which are activated with adenosine. Adenosine significantly reduces ipRGC responses by suppressing the cAMP-related pathway. Activation of A1 adenosine receptors inhibits the enzyme adenylate cyclase, which consequently leads to a decrease in intracellular cAMP levels. cAMP, in contrast, has been shown to increase the duration of light-evoked responses from the ipRGCs (Sodhi and Hartwick, 2014).

In addition to their connection with the dopaminergic amacrine cells, ipRGCs also project to non-visual areas, including the suprachiasmatic nucleus, which is involved in controlling circadian rhythms, and to the olivary pretectal nucleus, which plays a role in the pupillary light reflex response (Hattar, Lucas et al., 2003). The pupillary light reflex response has been defined as “the constriction of the sphincter pupillae muscles of the iris that is produced by an increase in retinal illuminance,” and there has been longstanding

evidence pointing to the involvement of luminance neurons in the pretectal olivary nucleus in mediating this response (Gamlin, Zhang et al., 1995). It was not until more recently that these "luminance neurons" have been identified as the ipRGCs.

Because ipRGCs express melanopsin, these cells have a peak spectral sensitivity of 478nm (Zhang et al., 2008). In addition to their distinct spectral sensitivity, ipRGC responses can be distinguished from those originating in rods and cones by the dynamics of their responses to light. ipRGCs exhibit relatively low light sensitivity compared to rods and cones, meaning that these cells need a much brighter light in order to reach threshold. Stimulation with brighter light evokes stronger responses from these cells (Graham and Wong, 1995). ipRGCs also produce a more sustained excitatory light response with a longer latency period when compared to rods and cones. These cells will continue firing even after the light stimulus has been turned off. This is different than the typical response of rods and cones, which respond quickly to a light stimulus by an increase in response frequency, but upon removal of the light stimulus, the rods and cones will show a rapid decline in the response rate (Sodhi et al., 2014). Additionally, ipRGCs will depolarize in response to light, which is very different from the typical hyperpolarization response that is seen in rod and cone photoreceptors (Zhang et al., 2008).

There is evidence that the pupillary response is at least partially driven by the intrinsically photosensitive retinal ganglion cells. As mentioned previously, one of the places the ipRGCs project to is the olivary pretectal nucleus, which plays a role in the pupillary light reflex response. Further, it has been shown that even humans who no longer have normal

vision due to the loss or the degeneration of rods and cones are still able to show pupillary constriction in response to bright lights (Zaidi, Hull et al., 2007). However, the ipRGCs are not the only cells contributing to the pupillary light response, as rods and cones also play a role in the normal retina. An animal study by Lucas et al. utilizing the mouse model showed that normal mice, mice without rod or cone function, and mice without functioning melanopsin within the ipRGCs all showed pupillary constriction upon exposure to bright light, as long as the defect was isolated to either the rods and cones, or to the ipRGCs. In melanopsin knock-out mice, the pupil still showed constriction in response to bright light, although the constriction was not as robust as compared to the response in the mice with complete and normal photoreceptor function. When the opposite was done, and the rod and cone photoreceptor function was knocked out, the pupil still constricted due to the response from the intact ipRGCs. However, when melanopsin, rods, and cones were knocked out, these mice showed no pupil constriction in response to bright light. This study showed that there is some redundancy in the circuit responsible for the pupillary light reflex and provided evidence for the idea that ipRGCs play an important role in pupillary responses to light (Hattar et al., 2003).

The pupillary light reflex is therefore the most readily quantifiable behavior driven by the ipRGCs, and their distinct firing pattern allows the responses from these cells to be distinguished from rod and cone input in the pupillary light reflex response (Lucas, Douglas et al., 2001). Because of their peak spectral sensitivity of 478 nanometers, ipRGCs are more sensitive to pulses of bright blue light. In contrast, ipRGCs are typically less sensitive to red light pulses. One way to measure the responses of the ipRGCs is to

measure the difference in pupillary responses to red compared to blue pulses of light. In addition to a higher level of constriction in response to blue light, the pupil also tends to remain constricted for a longer period of time (i.e., show slower redilation) following stimulation by blue light due to the prolonged post-illumination time course of the firing of ipRGCs (Zhang et al., 2008). Upon removal of the blue light stimulus, the pupil will re-dilate more slowly than what is seen with removal of the red light stimulus. This sustained dilation is often referred to as the "post-illumination pupil response" (Adhikari, Pearson et al., 2015).

In order to measure the input from the ipRGCs, researchers commonly utilize a pupillary testing protocol consisting of pulses of red and blue light in order to take advantage of the different spectral sensitivity characteristics of the ipRGCs compared to rod and cone photoreceptors. In a study conducted by Adhikari et al., the protocol for pupil response testing consisted of stimulus presentations of one second of red light, one second of blue light, ten seconds of red light, and ten seconds of blue light. Each stimulus pulse of light was preceded by a fifteen second interval: ten seconds of darkness and a during which the baseline pupil diameter was measured in darkness before onset of light pulse. Prior to any testing, participants underwent a ten-minute period of dark adaptation. As to be expected, blue pulses elicited a significantly larger post-illumination pupillary response compared to red pulses due to melanopsin having greater spectral sensitivity for short wavelength blue light. However, the researchers ultimately concluded that refractive error has no effect on the pupillary light response during light stimulation, or on the post-illumination pupillary response (Adhikari et al., 2015).

However, it is important to note that the total amount of light exposure was a mere twenty-two seconds for each round of pupillary testing, when accounting for both pulses of red and blue light. If the effect of dopamine comes from its diffusion throughout the retina, is this an adequate amount of stimulation and time to accurately assess ipRGC function? Another study conducted by Abbott, Queener, and Ostrin investigated the relationship between ipRGCs, refractive error, light exposure, and sleep. In this study, the ipRGC testing sequence consisted of a one-second pulse of red light, a sixty second measurement period, then a five second pulse of red light, followed by a sixty second measurement period. There was then another period of five minutes of dark adaptation before this sequence of light pulses and measurement periods were repeated using blue light. The study found that the amount of light exposure and time outdoors had an influence on morning melatonin concentration, but only when assessing the light exposure and time outdoors over the seven days prior to the assessment. However, no differences were found for morning melatonin concentration between refractive error groups. Refractive error also was not found to be associated with the ipRGC-driven pupil responses (Abbott, Queener et al., 2018).

The current study is designed to investigate some of the questions raised by the existing literature. Time outdoors has been consistently associated with delaying the onset of myopia, both in animal models and in human studies. The predominant explanation for this association is the role of visible light. The hypothesis is that increased light exposure leads to stronger responses from intrinsically photosensitive retinal ganglion cells, which

leads to increased release of dopamine throughout the retina. Dopamine is the retinal neurotransmitter that is responsible for the inhibition of axial elongation. The question remains that if increased robustness in response of the ipRGCs is related to increased release of dopamine in the retina, shouldn't ipRGC-related pupillary testing correlate to refractive error? The current study utilizes a pupillary testing protocol that has been used in previous research that found such a correlation. The protocol consists of longer exposure periods to red and blue light pulses, including one round of exposure to alternating pulses of red and blue light, one round of red-only pulses, and one round of blue-only light pulses. Subjects are dark adapted for five minutes before each of these three stimulus conditions. Although this testing protocol in the current experiment has the advantage of having shown correlations with refractive error, the disadvantage is the amount of time the pupillary testing protocol takes. Ultimately, the goal is to utilize pupillary testing protocols in children in order to detect those most at risk for the development of myopia or high myopia. In order to make the time needed for testing shorter and more feasible, the dark adaptation period in the current pupillary testing protocol would ideally be eliminated. Pupillary testing with the dark adaptation included in the testing takes over twenty minutes, while the testing without dark adaptation takes a mere five minutes. The purpose of the current study is to determine whether or not dark adaptation matters in the pupil testing protocol. It will also determine whether refractive error is related to pupillary light responses as measured with the current pupillary testing protocol and the briefer one without dark adaptation.

Chapter 2: Methods

Subject Recruitment

Approval for the study was obtained from the Biomedical Sciences Institutional Review Board (IRB) for The Ohio State University. Subjects were between the ages of 18 and 30 years. Myopia most often has its onset during the school years with peak incidence beginning at about age 9 that then falls off by the late teens (Blum, Peters et al., 1959; Kleinstein, Jones et al., 2003). Recruiting subjects between the ages of 18 and 30 years of age made it highly likely that subjects had already developed their full refractive error.

Following IRB approval, one e-mail was sent to current students of The Ohio State College of Optometry, and contact was made with twenty students who met the study criteria to schedule their appointments. These individuals were scheduled to complete five trials of experimentation. The sample size was chosen based on an estimate of repeatability and effect size. Repeated measures of two investigators were taken on different days. Their outcomes had a range of 0.12 (the units are normalized pupil response between 0 and 1.0; Beckett D, The Effect of Light Exposure and Refractive Error on Post-Illumination Pupil Responses. Unpublished Master's Thesis). The standard deviation of differences under the null hypothesis was conservatively estimated to be half

of this range (0.06). The desired confidence interval for estimating repeatability and the minimum effect size for evaluating different test conditions was set to ±0.03. Using this 2:1 ratio of the standard deviation to the confidence interval or effect size, a sample size of 16 subjects was calculated at an alpha = 0.05. To account for subjects who may not complete all sessions, the sample size was set at 20 subjects. Of the 20 individuals recruited, 11 were female and 9 were male.

Before beginning the study, all subjects signed written consent forms after being informed of the purpose of the study, the potential risks and benefits of the study, and the details of participating.

Exclusion/Inclusion Criteria

Upon the initial visit, in addition to obtaining written consent, subjects were also screened to ensure that the inclusion criteria were met. To be eligible for the study, participants had to be between the ages of 18 to 30 years old. Participants could have any refractive error, but they had to have a visual acuity with best correction no worse than 20/25 in either eye, as well as no active ocular or systemic health condition likely to affect pupillary response. A brief ocular history was taken to ensure no history of ocular disease such as glaucoma or cataracts, strabismus, sensitivity to anesthetics or dilating eye drops, refractive surgery, or a history of difficulty with pupillary dilation. One potential participant was excluded due to a history of strabismus surgery. Upon collecting this ocular history, no additional data was taken. This potential participant is not included in the 20 participants who were used for data analysis. A brief systemic health history

was also taken to ensure participants had no health conditions that would be likely to alter the pupil response. Systemic health conditions to be excluded included Marfan's syndrome, Down's syndrome, and diabetes. Included in the medical history was also a question as to whether the participant was pregnant or planning a pregnancy. The pupil testing presents no known risk to the mother or to the fetus, so no pregnancy testing was completed other than the initial health history.

Examination: Baseline Visit

Subjects participated in a total of 6 visits. The first visit was a baseline visit to obtain consent, medical history, refractive error, and to distribute a personal light-sensitive dosimeter used to monitor exposure to visible light. During this initial visit, participants were first given the written consent form, then were encouraged to read through and discuss the purpose of the study, the study's potential risks and benefits, and the testing procedure. After obtaining written consent from the participant, brief medical and ocular histories were collected with questions to determine eligibility. Questions also included age, gender, self-identified ethnicity, and parental history of refractive error. Distance visual acuity was tested and recorded for each eye using a Snellen visual acuity chart. The subject's non-cycloplegic refractive error in each eye was then assessed using the Grand Seiko WR-5100K. A Badal track was used along with the Grand Seiko WR-5100K to encourage relaxation of accommodation. This machine is a non-contact automated refractor that gives a printout of multiple refractive error readings. A minimum of five readings were taken on each eye.

Refractive error of each participant was further recorded as the average of the spherical equivalent of the two eyes. Spherical equivalent is equal to the sphere power added to half of the cylinder power. The spherical equivalent was calculated for each eye, then the average of these two values was recorded as the non-cycloplegic refractive error. Finally, participants were given a light sensing badge and verbal instructions to wear the light badge during all waking hours 24 hours prior to their next visit (Daysimeter, Lighting Research Center - Rensselaer Polytechnic Institute). These badges are currently used in numerous applications to examine entrainment of circadian rhythms, lighting ergonomics, and various aspects of the impact of lighting on human health. The badges are small and lightweight, water-resistant, attachable to clothing, have a battery life of up to 26 weeks, are capable of recording continuously every minute, and send data to a PC. Light exposure over these 24 hours served as a covariate during data analysis. Badges were not worn by participants while they slept, or while they were exposed to water (swimming, showering, etc.). Subjects were instructed to wear these light badges on their wrists during all waking hours 24 hours prior to their next study session. The badges were attached to wristwatch bands, and were easily secured. The study was conducted during the summertime, so the risk of participants covering the light badges with sleeves or other clothing was minimal. If for some reason long sleeves were worn, subjects were instructed to wear the light badges exterior to any articles of clothing. At the beginning of each pupil response testing session, subjects removed their light badge and the data were collected from each badge. The badges were then reset and returned to the participant to wear 24 hours before the next testing session. Upon receiving the badge, participants then scheduled their next study visit, at least 24 hours after the baseline visit.

Examination: 5 Trials of Pupillary Testing

After the initial visit to obtain consent and gather basic health and refractive error information, participants then underwent 5 sessions of pupillary testing. Testing sessions occurred on separate days to ensure that one condition did not affect another. This time frame also allowed for monitoring of light exposure in order to have light exposure serve as a covariate in data analysis. Pupil response to flashes of light was measured using an FDA-approved, commercial, video-based pupilometer (RAPDx; Konan Medical; www.rapdx.com). This instrument uses blue and/or red colors of light oscillating at a low temporal frequency of 0.1Hz to elicit a pupil response, and automatically records the pupillary responses in real time.

The five test sessions consisted of: 1) the standard pupillary testing protocol; 2) a repeat of the standard protocol; 3) the standard protocol without dark adaptation between lighting conditions; 4) the standard protocol with two minutes of red-only light (instead of the standard one minute) with dark adaptation and 5) the standard protocol with two minutes of the red-only light without dark adaptation. The standard protocol consisted of alternating flashes of red and blue light, followed by flashes of red-only light, and then by flashes of blue-only light (Figure 1). The testing session lasted approximately 30 minutes. Participants would undergo five minutes of dark adaptation between each of the three stimulus presentations whenever dark adaptation was used. Participants would sit in the testing room with all of the lights turned off, with no exposure to any of the light outside the testing room, and only exposure to the dim ambient light from the RAPDx

pupilometer. Then, participants were placed in front of the RAPDx pupilometer, which presented LED-based light stimuli to both eyes in order to elicit a pupillary response.

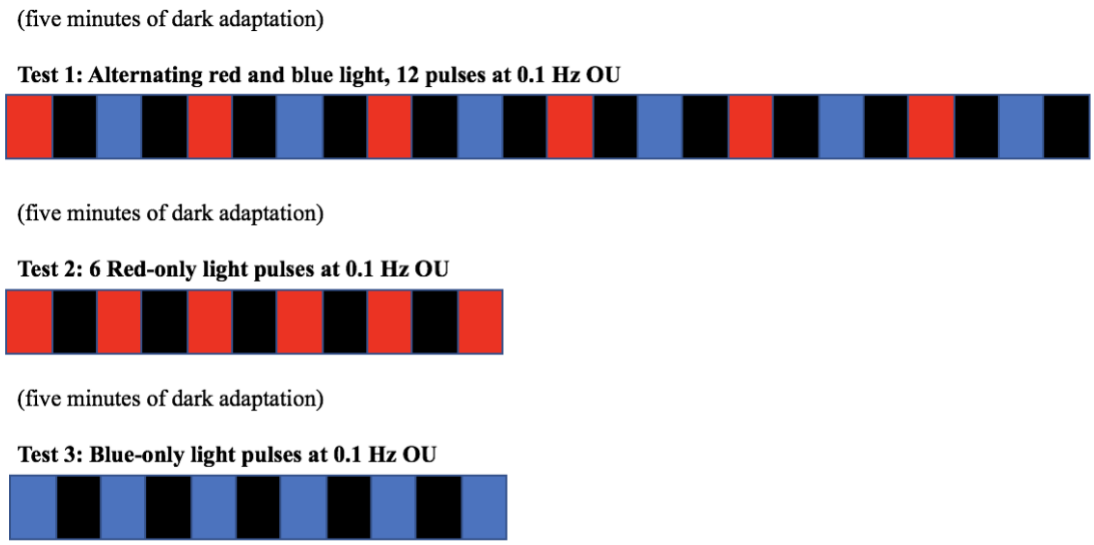

Figure 1: The standard pupillary testing protocol

During this testing, participants were instructed to try to refrain from blinking until two seconds after the flash of light had been presented, in order to obtain the full recording of the pupil response. For the first exposure to flashes of light, participants were presented with alternating flashes of red and blue light. Participants would see 5 seconds of red light, followed by 5 seconds of darkness, then 5 seconds of blue light, followed by 5 seconds of darkness (0.1 Hz oscillation). In total, participants were presented with 12 pulses of alternating red and blue lights. Participants underwent another five minutes of

dark adaptation if dark adaptation was used, were placed back in the RAPDx pupilometer, and were then presented with 6 pulses of red light only. The timing interval was the same: five seconds of red light followed by 5 seconds of darkness. Participants underwent one final round of five minutes of dark adaptation if dark adaptation was used, then were placed back in the RAPDx pupilometer, and were then presented with 6 pulses of blue only light. The timing interval remained the same: five seconds of blue light followed by 5 seconds of darkness. Two of the five sessions consisted of the standard testing protocol, in order to determine its repeatability.

Dark adaptation increases the time needed to complete the protocol. In order to determine the effect of dark adaptation on the pupil responses, one session consisted of the standard protocol but without dark adaptation. During this testing session, participants were placed in the RAPDx without first dark adapting and then were exposed to the alternating pulses of red and blue lights, the pulses of red lights, and then finally the pulses of blue lights without ever sitting back from the RAPDx.

A fourth testing session consisted of longer exposure to the red-only pulses of light. The testing protocol was the same as the standard protocol, except that the red-only pulses of light lasted for two minutes (12 five-second pulses of light each followed by five seconds of darkness). In this testing condition, the exposure to blue pulses of light remained the same as in all other testing conditions. The purpose of the extended red presentation condition was to determine if greater pupil responses to blue would result due to photopotentiation created by the longer exposure to red.

Finally, the fifth testing protocol still consisted of the same sequence of alternating lights, the two minutes of red-only pulses, and the standard pulses of blue light. However, in this condition no dark adaptation occurred in order to determine its effect. Once the participant was placed in front of the RAPDx pupilometer, the participant did not sit back until the entire testing sequence was complete.

The order in which participants underwent these various testing conditions was randomized to ensure that the order these protocols were presented did not have an effect on pupillary response. The first four sessions of pupillary testing were scheduled to last approximately 30 minutes (although the protocols without dark adaptation often were completed more quickly). The final visit of the study was intended to last approximately one hour. During the final visit for the study, participants completed one last measure of refractive error. After completing the fifth and final round of pupillary testing using the RAPDx, participants were given one drop of proparacaine 0.5% as an ocular anesthetic, then two drops of tropicamide 1% as a mydriatic and cycloplegic. Cycloplegic autorefraction was completed using the Grand Seiko WR-5100K 25 minutes after the instillation of the eye drops. Upon completion of this autorefraction, participants had finished all parts of the study. Participants were compensated after each study session, and signed documentation indicating that they had received compensation for participation.

Statistical Analysis

All subject data was compiled in one master Excel sheet. This sheet contained the subject's coded identity along with pertinent data from the initial baseline visit. Such data included age, self-identified ethnicity, parental refractive error and age of onset, refractive error age of onset, and both non-cycloplegic refractive error and cycloplegic refractive error as measured with the Grand Seiko WR-5100K (recorded as the average spherical equivalent of the two eyes). In addition to this information, the Excel spreadsheet also contained information from each of the five testing sessions, including light badge data as well as pupillary response data. At the beginning of each session, data from the light badge was imported into an Excel document. Data were parsed out to only include readings collected 24 hours prior to the study session appointment. The lux values recorded by the light badge over 24 hours were summed, then the base 10 log of this value was taken in order to determine a final number to record in the master sheet to quantify light exposure. Log-lux values were used to make the skewed distribution for raw light exposure closer to normally distributed data. This was done for each of the five testing sessions. The light badge was then reset before returning it to the participant.

After each round of RAPDx pupillary testing, the data collected from the pupilometer were retrieved from the machine and transferred to the computer. The data were imported into an Excel sheet, which calculated pupillary constriction and dilation in response to alternating flashes of red and blue light, to the red-only flashes of light, and to the blue-only flashes of light for the entire duration of the testing. In order to account for inter-subject and inter-session variation in baseline pupil size, pupil data were normalized for a

given subject across the testing conditions within each of the five test conditions. The normalization equation was as follows: normalized pupil size = (maximum pupil size – pupil size)/(maximum pupil size – minimum pupil size). Maximum constriction (i.e., minimum pupil size) was considered to be 100%, and maximum dilation was considered to be 0%. Values resulting from blinks were also accounted for. Any value that produced a "zero," "divided by zero," or an obscure negative number that was the result of a participant blinking was removed. All values were screened both by the Excel program and by hand to ensure the removal of all data contaminated by blinks. For each time interval, data from the right and left eye were averaged to give one overall value for pupillary response. Then, the value for the response to the blue flash of light during the alternating light sequence was subtracted from the value that corresponded to the same time interval for the blue only flashes of light. The differences were calculated for each time interval, then summed and averaged to give the average difference between the pupillary response to the blue flash of light during the alternating light sequence and the pupillary response to the blue flash of light during the blue-only sequence. The same was done for the red flashes of light. These two values were recorded in the master excel sheet, and were used for further statistical analysis.

Data analysis was conducted using SPSS version 24. Results were considered significant at $p < 0.05$.

Chapter 3: Results

All twenty subjects completed the screening visit, as well as five trials of pupillary testing. Data collected from all participants included refractive error, daily light exposure, and pupillary responses to flashes of red and blue light over the five testing sessions. Descriptive statistics were collected on the entire sample size (n = 20). The subjects' mean age was 24.0 ± 2.37 years. There were a total of 11 females and 9 males enrolled in the study. Participants of any refractive error were included, but the majority of subjects were myopic. The mean cycloplegic refractive error was -3.22 ± 2.78 D. Refractive errors ranged from -10.61 to +0.77 D.

The mean and standard deviation for each pupillary testing condition are summarized in Table 1. The outcome measure was defined as the difference in pupillary response to the single color flash of light (mono) minus the pupillary response to the alternating flash of light (alt) of the same color for corresponding time periods.

Condition	Mono – Alt Blue ± SD	Mono – Alt Red ± SD
Standard 1	0.097 ± 0.03	-0.005 ± 0.06
Standard 2	0.105 ± 0.03	-0.010 ± 0.05
Standard No DA	0.069 ± 0.03	-0.011 ± 0.04
Long Red	0.105 ± 0.04	0.000 ± 0.05
Long Red No DA	0.081 ± 0.04	0.001 ± 0.04

Table 1: Descriptive Statistics for Pupillary Responses to Blue and Red Light Under Each of the Five Conditions. DA = dark adaptation

Table 2 shows the estimate of repeatability for the standard pupillary testing protocol. As seen in this table, there was no statistically significant difference between the pupillary response measure for standard condition 1 compared to standard condition 2 when looking at the pupillary responses to the flashes of single or alternating blue light. The mean difference column is the mean outcome measure of pupillary response (single color blue minus the alternating blue light response) under the standard condition 1 minus the mean outcome measure of pupillary response under the standard condition 2.

	Mean Difference (± SD) Standard 1 – Standard 2	**95% Limits of Agreement**	**p-value**
Mono – Alt Blue	-0.0075 ± 0.040	±0.078	0.41
Mono – Alt Red	0.0043 ± 0.067	±0.13	0.77

Table 2: Pairwise Comparisons of the Mono – Alternating Blue and Mono – Alternating Red pupillary responses for the Two Standard Testing Conditions

Figure 2 shows a graph of the pupillary response outcome measures under the five testing conditions. “DA” denotes whether or not the participant underwent “dark adaptation” during a particular testing condition. “Long Red” signifies the condition under which participants were exposed to an extra minute of red light. The points at the top of the figure represent the pupillary response outcome measure to blue light, and the points at the bottom of the figure represent the outcome measure to red light. The blue arrows represent conditions which showed a significant difference in pupillary response outcome

to the blue flashes of light. Consistent with the data shown in Table 2, there was no statistically significant difference in the outcome measures between the first and second trials of the standard condition. The graph also shows that there was a statistically significant difference in the outcome measures of pupillary response to blue light for the first presentation of the standard condition compared to the standard condition with no dark adaptation, the second presentation of the standard condition compared to the standard condition with no dark adaptation, and the long red condition with dark adaptation compared to the standard condition with no dark adaptation. None of the conditions produced any significant differences in pupillary response to the single color minus alternating red light outcome.

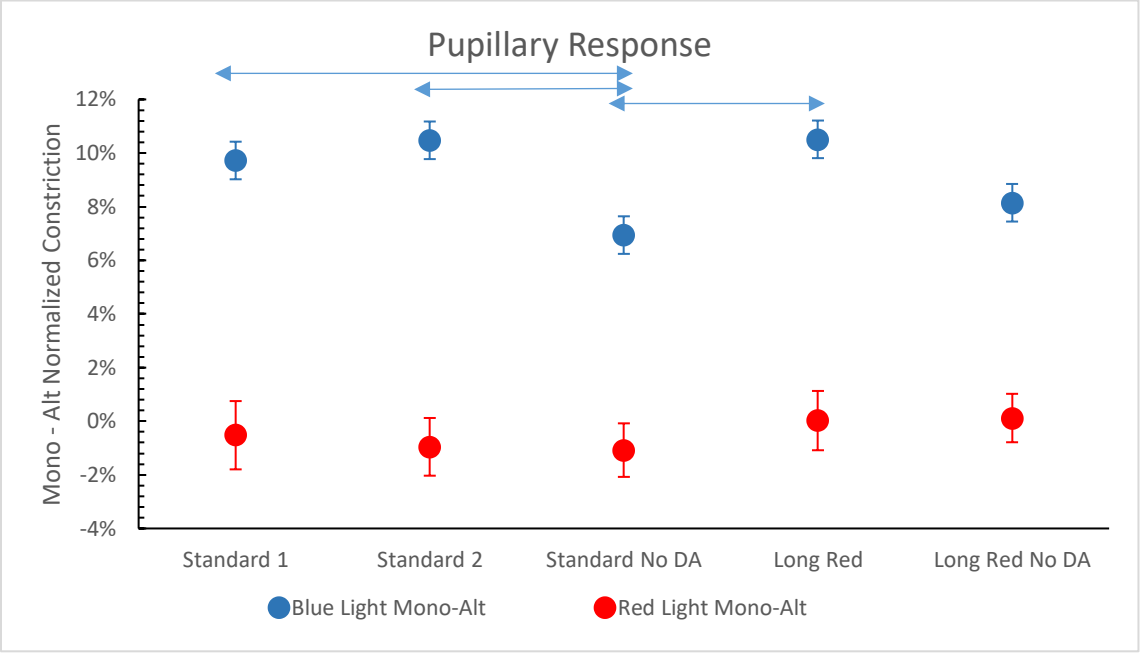

Figure 2: Mono – Alt Pupillary Response Outcome Measures Under the Five Testing Conditions. DA = dark adaptation; "Long Red" = two minutes of exposure to red as a single color. The arrows indicate significant differences between conditions.

These pair-wise comparisons are also shown in Table 3. The first column represents the condition that is being examined, and the second column represents the condition to which it is being compared. The mean difference column is the mean outcome measure of pupillary response (single color minus the alternating light response) under the condition in column one minus the mean outcome measure of pupillary response under the condition listed in the second column. There was no significant difference between pupillary responses under the two standard testing conditions (mean difference = -0.008 ± 0.040). Because of this, the two standard conditions were combined into one average outcome during the pairwise comparisons. As seen in Figure 2 and Table 3, for the single color minus alternating blue pupillary response measure, there were significant differences between the standard condition and the standard condition with no dark adaptation, as well as between the long red condition which included dark adaptation, and the standard condition with no dark adaptation. It is also of note that the difference between pupillary response measures during the long red with dark adaptation when compared to the long red condition without dark adaptation did not appear to be statistically significant ($p = 0.052$), as might have been expected based on the results of the standard conditions with and without dark adaptation. These pairwise comparisons were also examined using a repeated measures analysis of variance, with dark adaptation and the length of the red stimulation as the repeated factors.

Condition	Condition Compared	Mean Difference ± SD	p-value
Average Standard	Standard No DA	0.032 ± 0.034	0.0030
Average Standard	Long Red	-0.004 ± 0.032	1.000
Average Standard	Long Red No DA	0.020 ± 0.045	0.40
Standard No DA	Long Red	-0.036 ± 0.032	<0.0001
Standard No DA	Long Red No DA	-0.012 ± 0.034	0.76
Long Red	Long Red No DA	0.024 ± 0.036	0.052

Table 3: Pairwise Comparisons of the Mono – Alternating Blue pupillary response outcome measure for the Testing Conditions. P-values are corrected for multiple comparisons (Bonferroni).

The effect of dark adaptation and the length of exposure to red on mono-alternating blue pupillary responses was further analyzed using a repeated measures ANOVA. No interaction was found between dark adaptation and the long red condition with respect to their association with the mono-alternating blue pupillary response (p value for interaction = 0.39); only the main effects are presented in Table 4. There was a statistically significant difference in pupillary response outcomes depending on whether or not the participant underwent dark adaptation. Regardless of the length of exposure to red as a single color, dark adaptation matters. However, when looking at the pupillary responses under the long red condition once adjusted for dark adaptation, it can be seen

that there is no effect of the longer exposure to red. Ultimately these tables show that dark adaptation does produce a statistically significant difference in pupillary responses whereas lengthening exposure to red light does not.

Conditions Compared	Mean Difference	Standard Error	p-value
Dark Adaptation vs. No Dark Adaptation	0.028	0.006	<0.0001
Long Red vs. No Long Red	0.008	0.006	0.18

Table 4: Effects of Dark Adaptation and Increased Length of Exposure to Red Light Pulses

Another analysis was done for mono-alternating red pupillary responses. The first column of Table 5 represents the condition that is being examined, and the second column represents the condition to which it is being compared. The mean difference column is the mean outcome measure of pupillary response (single color minus the alternating light response) under the condition in column one minus the mean outcome measure of pupillary response under the condition listed in the second column. As shown in this table, there were no significant differences seen for pupillary responses to red light under the different testing conditions. Unlike the differences seen for blue light responses in Table 3, there was no difference in pupillary responses to red light between the average standard condition when compared to the standard condition with no dark adaptation.

Condition	Condition Compared	Mean Difference ± SD	p-value
Average Standard	Standard No DA	0.003 ± 0.062	1.000
Average Standard	Long Red	-0.008 ± 0.046	1.000
Average Standard	Long Red No DA	-0.009 ± 0.060	1.000
Standard No DA	Long Red	-0.011 ± 0.047	1.000
Standard No DA	Long Red No DA	-0.012 ± 0.036	0.935
Long Red	Long Red No DA	-0.001 ± 0.055	1.000

Table 5: Pairwise Comparisons of the Mono – Alternating Red pupillary response outcome measure for the Testing Conditions. P-values are corrected for multiple comparisons (Bonferroni).

Figure 3 shows the average pupillary responses to flashes of blue light during the period of alternating red and blue lights, versus the responses during the blue-only flashes of light under the first standard testing condition. “Mono Blue” in this figure represents the pupillary responses to light during the period of blue light-only pulses. “Alt Blue” represents the pupillary responses to light during the pulses of blue light within the alternating light sequence. This graph, as well as all graphs that follow, shows pupil constriction towards the top of the graph and pupil dilation towards the bottom. Pupil constriction was expected during the flashes of light and pupil dilation was expected

during the periods of darkness. This figure shows that during the blue-only flashes of light, the pupil was more constricted in response to the first flash of light, and remained more constricted to each flash of light throughout the entire testing sequence compared to alternating red and blue flashes. When looking at the periods of darkness, the pupil also remained more constricted after each of the blue-only flashes of light versus after the alternating flashes of red and blue lights. The pupillary response during the alternating light flashes showed less constriction during both periods of light and dark exposure. However, the pupil tended to become slightly more constricted with increasing number of light pulses under the alternating light flashes.

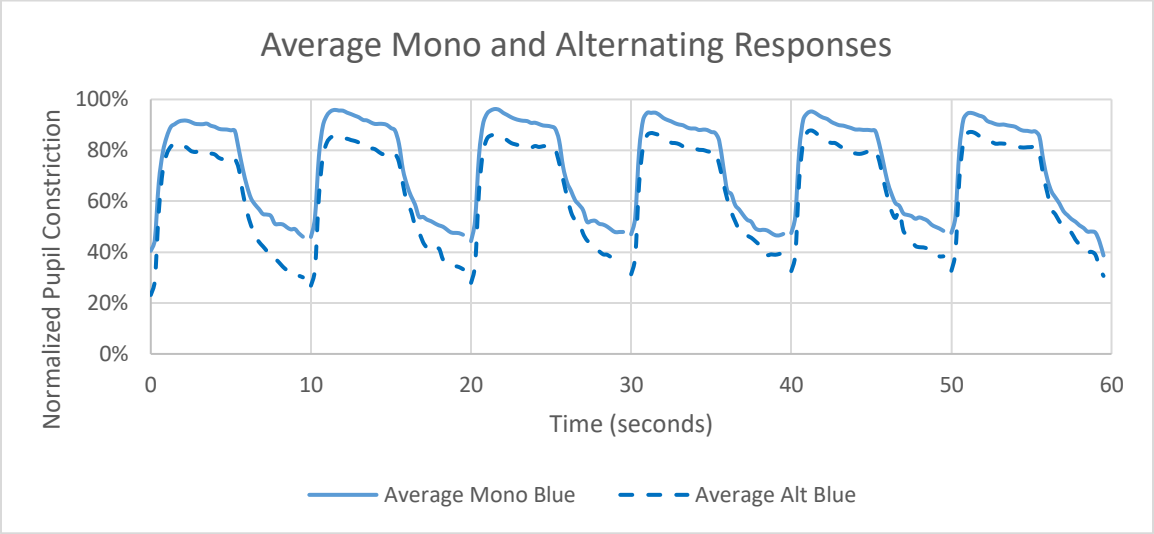

Figure 3: Pupillary Response to Flashes of Blue Light Under Alternating versus Blue-Only Light under the First of the Standard Testing Conditions with Dark Adaptation

Figure 4 shows the pupillary responses to alternating flashes of blue light compared to responses to the blue-only flashes of light under the standard condition with no dark adaptation. Compared to Figure 3, the pupillary response to the flashes of alternating blue light in Figure 4 more closely follows the pupillary response to the single color blue-only flashes of light. The response to the alternating light pulses remained similar to the standard condition responses, while the single-color blue responses were less constricted under the standard condition with no dark adaptation, although these were still more constricted than the pupillary responses to blue light during the period of alternating light flashes.

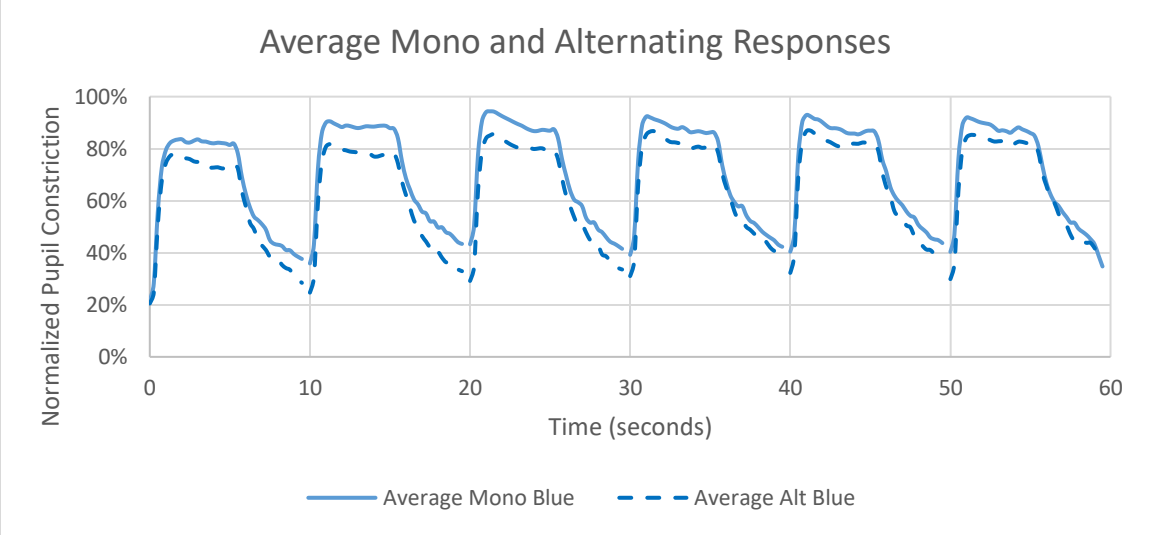

Figure 4: Pupillary Response to Flashes of Blue Light Under Alternating versus Blue-Only Light under the Standard Testing Condition without Dark Adaptation

Figure 5 shows the pupillary responses to the single-color flashes of red and blue light under the first standard testing protocol. “Mono Blue” represents the pupillary responses

during the flashes of blue light, whereas “Mono Red” represents the pupillary responses during the flashes of red light. Changing the light stimulus to single colors altered the pupillary response. Under the red-only light condition, the pupil size tended to get larger as the number of pulses increased. However, when looking at the responses to blue light only, the pupil tended to be smaller and to maintain a similar response as the number of light pulses increased. The more prolonged constriction (slower redilation) of the pupil during darkness under the blue light condition can be appreciated by looking at the difference between the red and blue graphs in Figure 5 during the periods of darkness. Additionally, the constrictions during the later pulses of the single-color red light showed faster, greater rebound dilation when compared to the constrictions to blue light and to earlier pulses of red light.

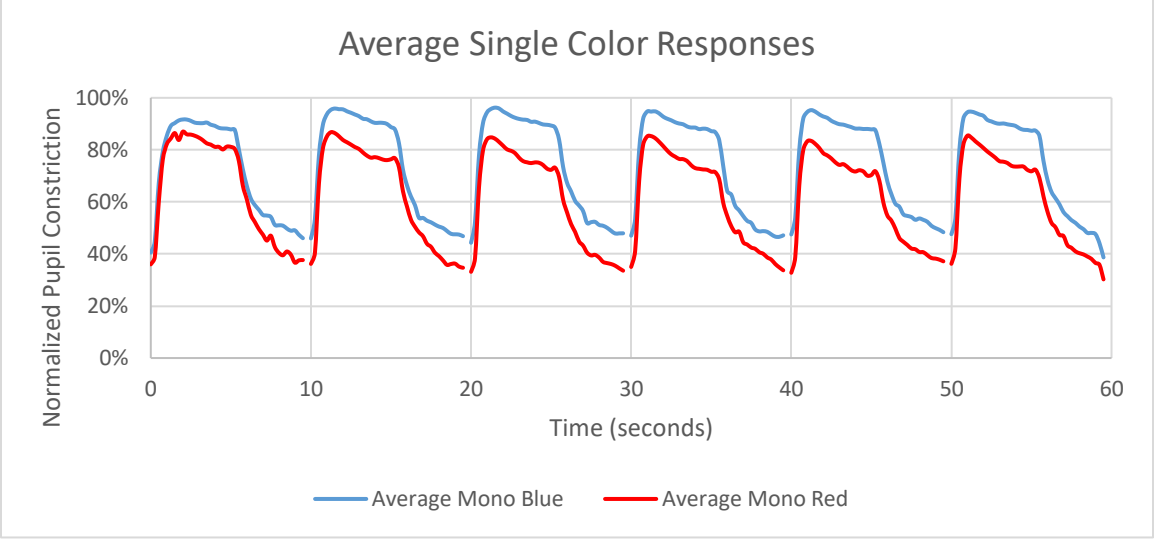

Figure 5: Pupillary Response to Single Color Flashes of Red or Blue Light under the First Standard Testing Condition with Dark Adaptation

The effect of dark adaptation on the pupillary response to blue as a single color can be seen in the comparison between Figures 5 and 6. Figure 6 depicts the pupillary responses to the flashes of either single-color red or blue light under the standard testing condition without dark adaptation. The blue light responses in Figure 6 are less constricted when compared to the responses seen in Figure 5, during the flash of light as well as during the period of darkness. In Figure 6, the blue responses started out at around 84% constriction and reach a maximum value around 94% constriction. Compare this to the blue responses to the standard condition with dark adaptation, in which the blue responses started at around 91% constriction and reached a maximum constriction of around 96% constriction. When looking at the periods of darkness between each blue light pulse, the responses under the standard condition with no dark adaptation dilated to around 40%, whereas the responses under the standard condition with dark adaptation dilated to around 50%.

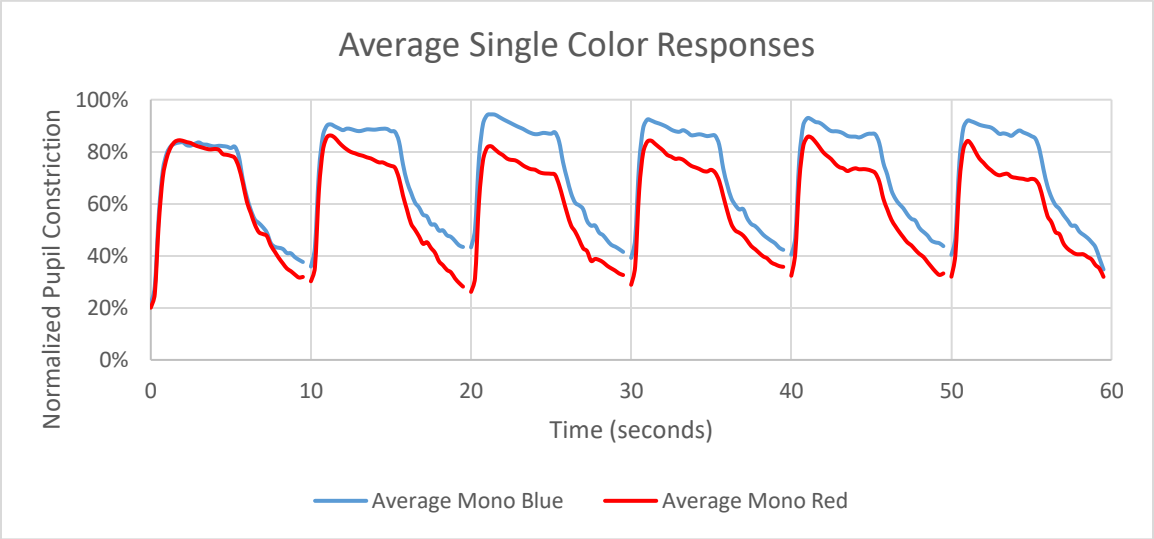

Figure 6: Pupillary Response to Single Color Flashes of Red or Blue Light under the Standard Testing Condition without Dark Adaptation

Figure 7 shows pupillary responses to flashes of alternating red and blue lights under the first standard testing protocol. The data depicted in the graph represents an average of the pupillary responses at each time point under each of the standard testing conditions. With the alternating flashes of light, the responses to the red light and to the blue light closely followed each other. As the number of pulses increased, the amount of pupillary constriction also increased.

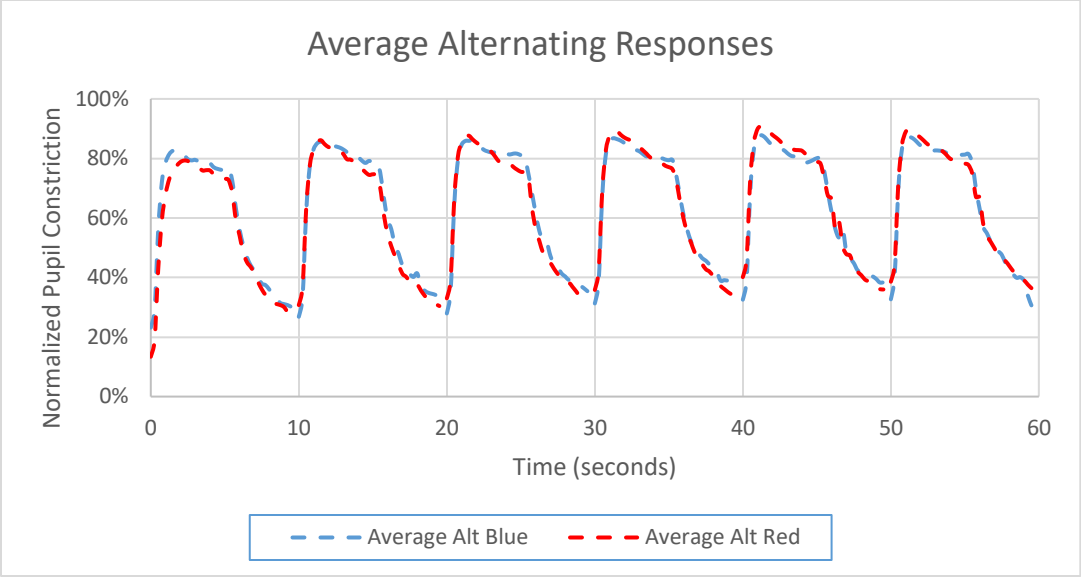

Figure 7: Pupillary Response to Alternating Flashes of Red and Blue Light under the First Standard Testing Conditions with Dark Adaptation

Figure 8 depicts pupillary responses to red and blue light under the alternating light condition during the standard testing protocol with no dark adaptation. The pupillary response under the alternating light condition was similar to what it was under the standard testing protocol which included dark adaptation (see Figure 7). The responses to the different colors of light still seemed to follow each other.

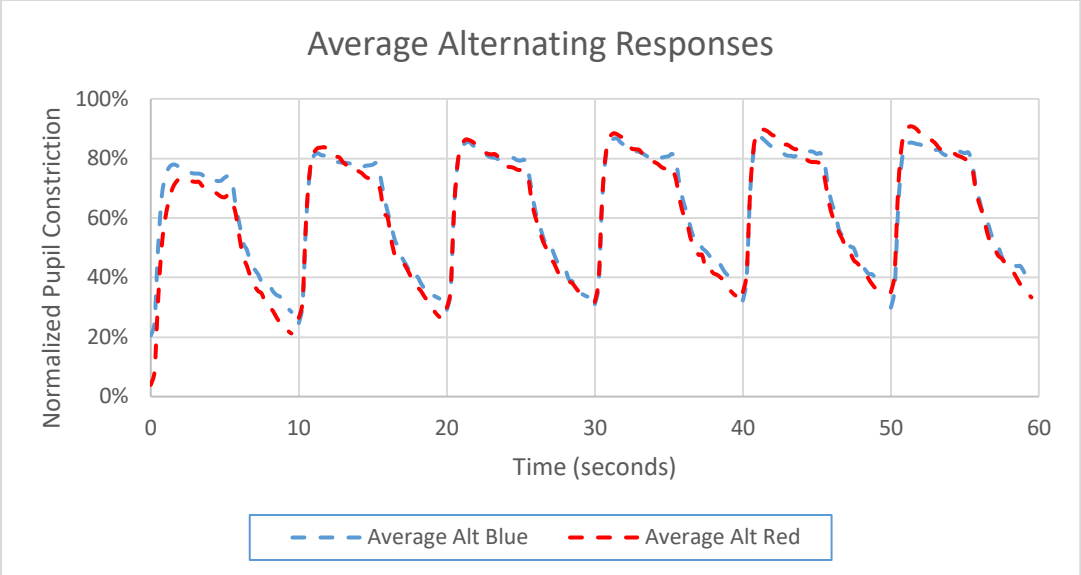

Figure 8: Pupillary Response to Alternating Flashes of Red and Blue Light Under the Standard Testing Condition without Dark Adaptation

Figure 9 shows the pupillary responses to the alternating flashes of light under the testing condition with the lengthened exposure to red light (two minutes of 0.1 Hz oscillating red light pulses as opposed to one minute) and with dark adaptation. The pupillary responses follow a similar pattern to that shown in Figures 7 and 8, with a slight reduction in the maximum pupillary constriction during the pulses of light.

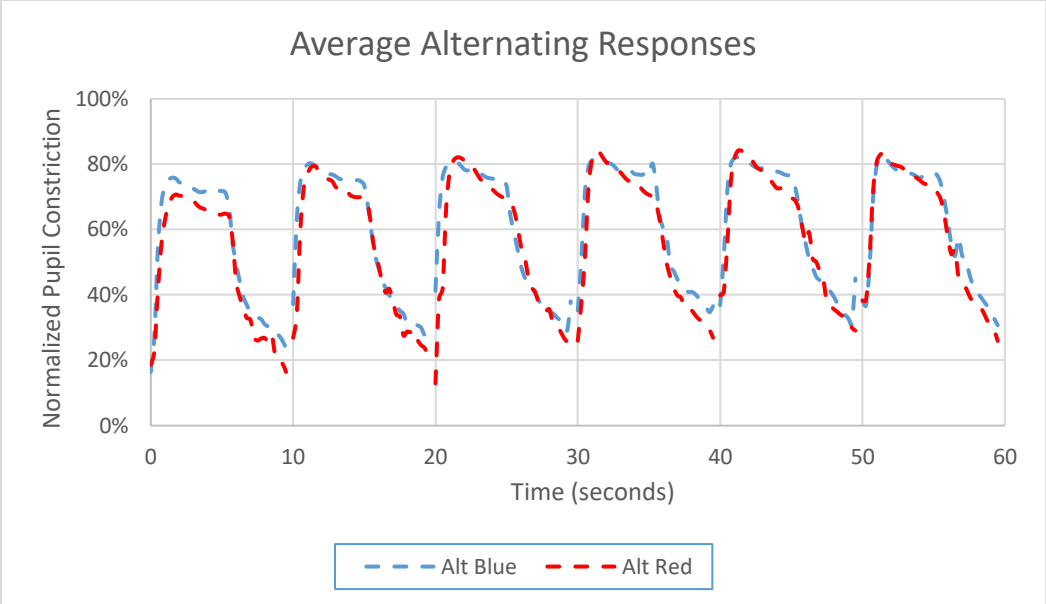

Figure 9: Pupillary Response to Alternating Flashes of Red and Blue Light Under the Long Red Testing Condition with Dark Adaptation.

Figure 10 shows the pupillary responses during the time periods in which only one color of light was shown. There are twice as many red pulses due to the fact that the red only light testing sequence was twice as long. The pupillary response was more constricted in response to the earlier flashes of blue light than it was during the flashes under the standard condition with no dark adaptation (see Figure 6). However, by the end of the light sequence, there was little difference in pupil constriction under the two conditions. The pupillary response was also more constricted in response to blue, as shown in Figure 10, during the periods of darkness in between pulses at about 50%, when compared to responses to blue shown in Figure 6 at about 40%. As shown in Figure 2, the pupillary response measures for blue light under the standard condition with no dark adaptation was significantly different compared to the testing condition with dark adaptation and the longer period of red light pulses.

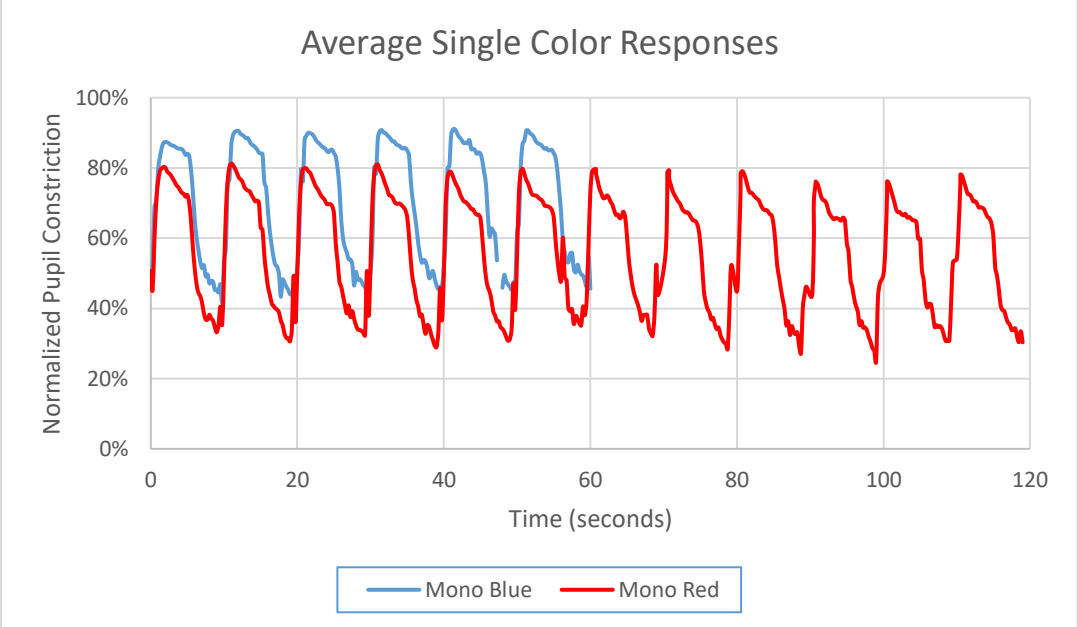

Figure 10: Pupillary Response to Single Color Flashes of Red or Blue Light under the Long Red Testing Condition with Dark Adaptation

Figure 11 shows the pupillary responses to alternating flashes of blue light compared to responses to the blue-only flashes of light under the long red testing condition with dark adaptation. The response to the blue light during the alternating light pulses remained similar across all testing conditions. However, the response to the blue-only pulses showed more constriction when compared to the response under the standard condition with no dark adaptation, especially when looking at the pupil constriction during the periods of darkness. Under the standard condition with no dark adaptation (Figure 6), the pupil redilated to about 40% during the periods of darkness, however, under the condition with longer exposure to red light with dark adaptation, the pupil constriction remained around 50% during periods of darkness following the single-color blue pulses of light (Figure 11). As was shown in Table 4, dark adaptation resulted in a smaller pupil in

response to blue light pulses, whereas longer exposure to red seemed to have little effect on pupil response.

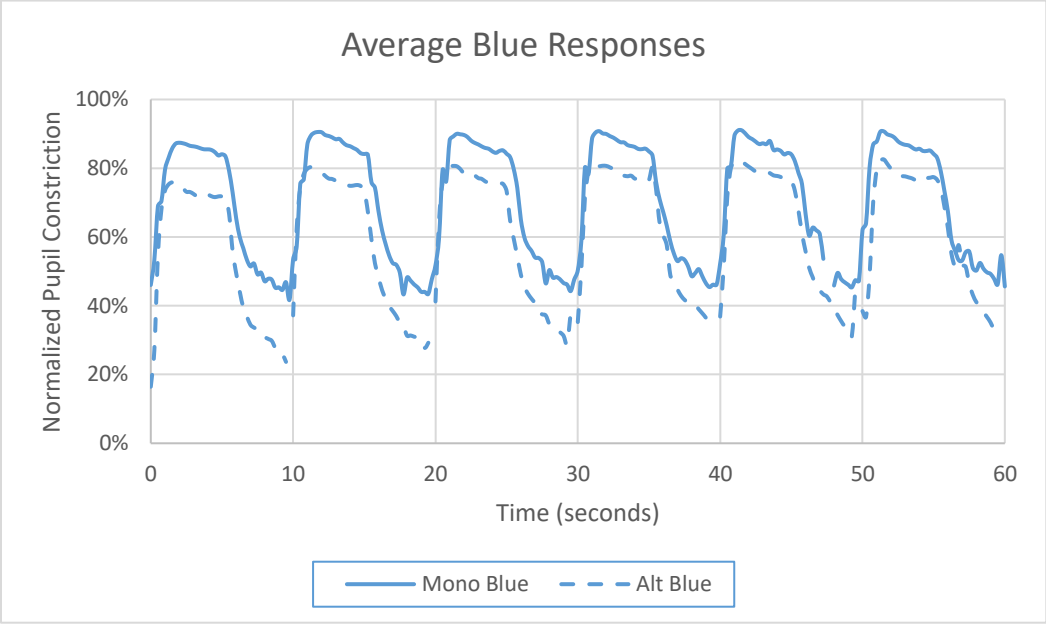

Figure 11: Pupillary Response to Flashes of Blue Light Under Alternating versus Blue-Only Light under the Long Red Testing Condition with Dark Adaptation

In comparison to Figure 11, Figure 12 shows the pupillary responses to alternating flashes of blue light compared to responses to the blue-only flashes of light under the long red testing condition with no dark adaptation. Similar to what was seen when comparing the standard testing condition with dark adaptation to the standard testing condition without dark adaptation, a comparison of Figures 11 and 12 show that the single color blue light elicits a less constricted response under the long red testing condition without dark adaptation. The pupillary response to the blue light pulses under the alternating light sequence appears similar in both figures.

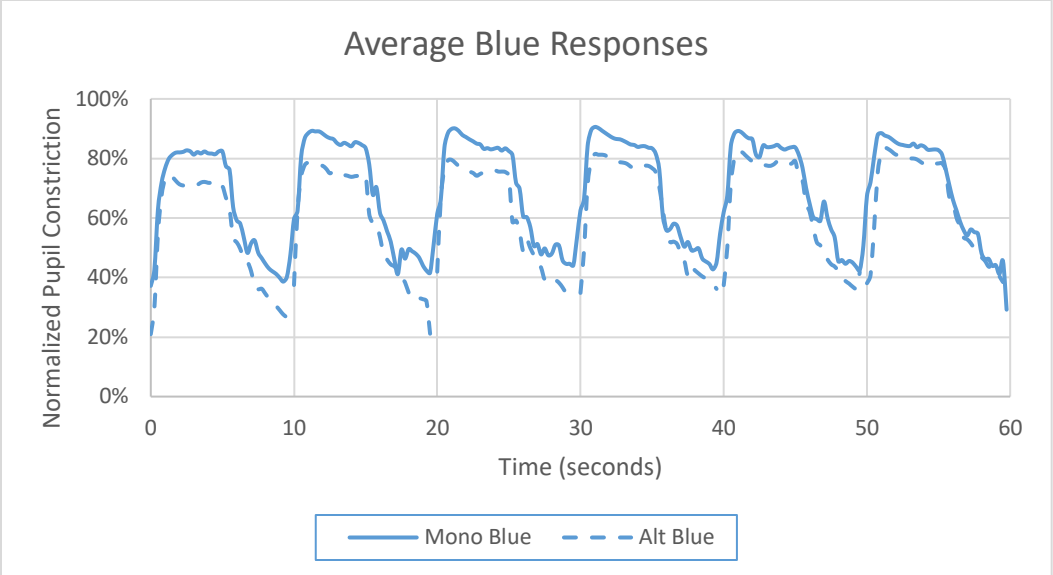

Figure 12: Pupillary Response to Flashes of Blue Light Under Alternating versus Blue-Only Light under the Long Red Testing Condition without Dark Adaptation

Figure 13 shows the pupillary responses to the flashes of red light during the alternating time period as well as to the single color red flashes during the long red testing condition with dark adaptation. As initially seen in Figure 2 and Table 5, there were no statistically significant differences in pupillary responses to red light between any of the five conditions.

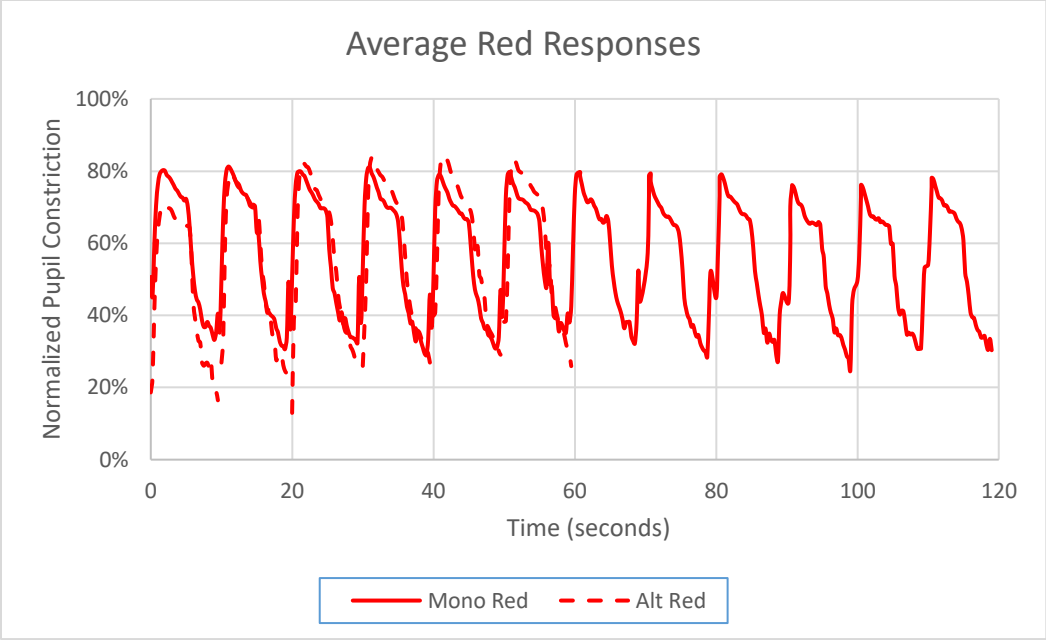

Figure 13: Pupillary Response to Flashes of Red Light Under Alternating versus Red-Only Light under the Long Red Testing Condition with Dark Adaptation

Figure 14 shows the pupillary responses during the time period of alternating flashes of red and blue light under the testing condition that included a lengthened exposure to single color red light pulses in addition to no dark adaptation. As before, the response is similar to the conditions in which dark adaptation was included, as seen when comparing Figure 14 to Figure 9.

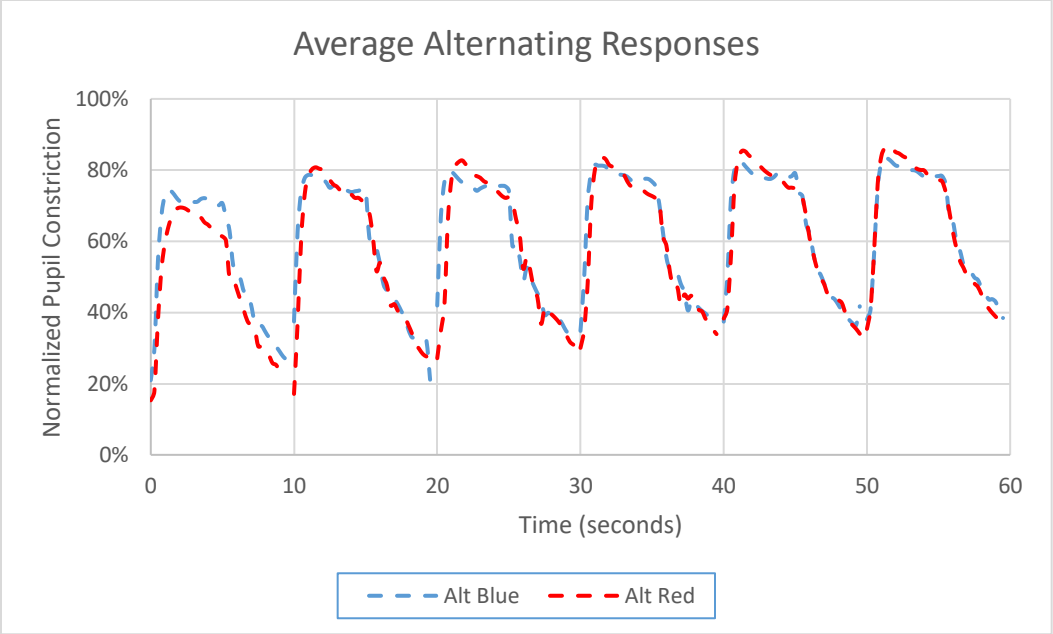

Figure 14: Pupillary Response to Alternating Flashes of Red and Blue Light Under the Long Red Testing Condition with no Dark Adaptation

Figure 15 shows the pupillary responses to single color flashes of light during the long red testing condition that did not include the periods of dark adaptation. The pupillary response tended to be a little less constricted during the period of blue light flashes, especially during the periods of darkness, when compared to Figure 10.

Figure 15: Pupillary Response to Single Color Flashes of Red or Blue Light under the Long Red Testing Condition with no Dark Adaptation

Figure 16 shows the average pupillary responses to flashes of red light during the period of alternating red and blue lights, versus the responses during the red-only flashes of light. During the first pulse of red light, the average pupillary response was more constricted in response to the red-only flash compared to the very first red flash of light that began the testing during the sequence of alternating red and blue lights. However, as the number of pulses of light increased, the response to the red flash during the alternating condition became more constricted when compared to the pupillary response to the red-only flashes of light. The response to the red-only flashes during both the actual light pulses themselves as well as the response to the periods of darkness remained relatively stable over the testing time period. Figure 16 shows that pupillary constriction was initially greater in response to the single color red light, but after the third pulse of light the pupillary constriction became greater in response to the pulses of red light in the

alternating red sequence. Further, this figure shows increased rebound during constriction when comparing later pulses to earlier ones in the single color red sequence. Comparing pulse 6 to pulse 1, Figure 16 shows this rebound in the steeper slope at the top of the graph for pulse 6, showing initial constriction quickly followed by rebound re-dilation during the exposure to red light.

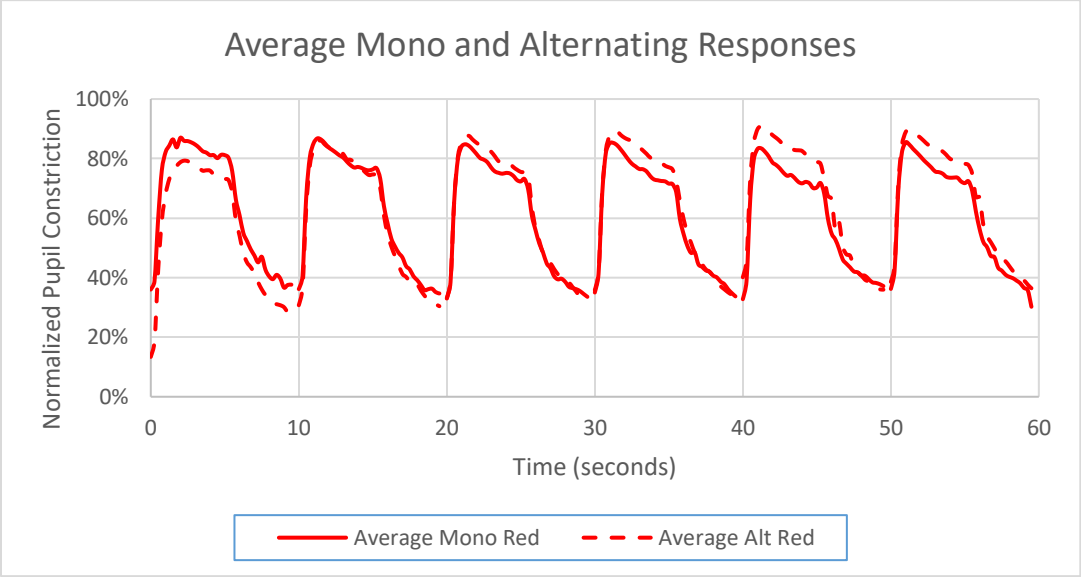

Figure 16: Pupillary Response to Flashes of Red Light Under Alternating versus Red-Only Light Conditions under the First Standard Testing Conditions

Figure 17 shows the pupillary responses to the flashes of red light during the alternating light time period as well as to the single color of red flashes during the standard condition with no dark adaptation. The responses show a similar pattern to what is seen in Figure 16. As seen in Figure 2 and Table 5, there was no statistically significant difference in pupillary responses to red light between any of the five conditions.

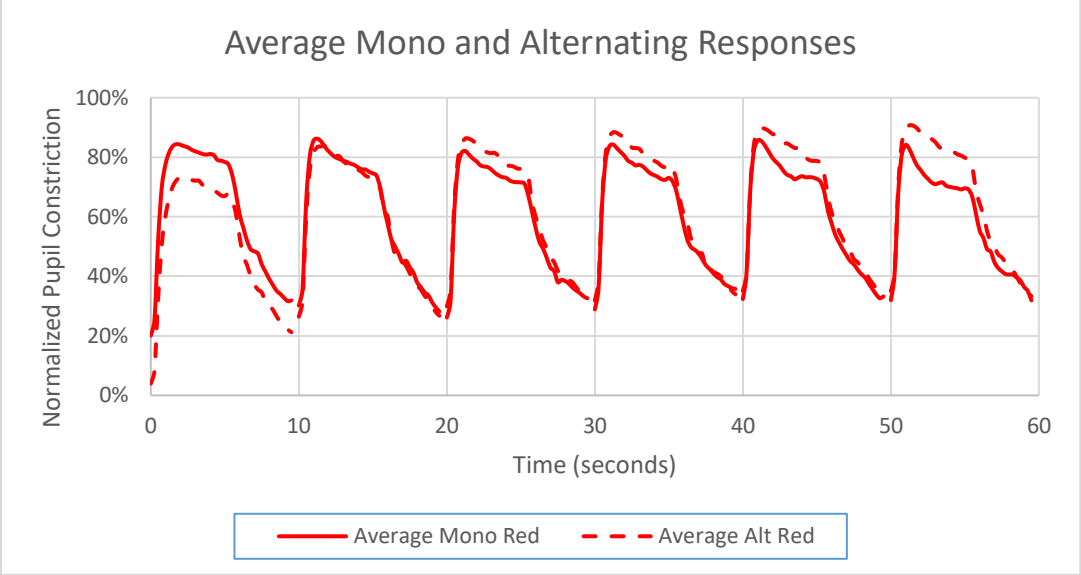

Figure 17: Pupillary Response to Flashes of Red Light Under Alternating versus Red-Only Light under the Standard Testing Condition Without Dark Adaptation

The pupillary response to red light tended to have a consistent pattern across all of the stimulus conditions. During the first pulse of red light, the average pupillary response was more constricted in response to the red-only flash compared to the red flash of light during the early portion of the sequence of alternating red and blue lights. However, as the number of pulses of light increased, the response to the red flash during the alternating condition became more constricted when compared to the pupillary response to the red-only flashes of light. As seen in Figures 16 and 17, there was a switch from the red-only flash of light eliciting the more constricted pupillary responses to the red light in the alternating sequence eliciting the more constricted pupillary responses. This switch was first seen during the third pulse of red light. The amount of difference appeared to be greater without dark adaptation in Figure 17 compared to with dark adaptation in Figure 16. Due to this difference, an analysis was conducted to determine whether there was a

significant difference between pupillary response to red light during the first light pulse compared to the pupillary response to red light during the sixth and final pulse that was related to dark adaptation. This single color minus alternating response measure from the sixth pulse of red light was subtracted from the single color minus alternating response measure from the first pulse of red light. The difference between the first and the sixth pulses was then compared between the standard condition with dark adaptation to without dark adaptation. As seen in Table 6, although the difference was close with a p-value of 0.064, the results did not show a statistically significant difference between dark adaptation and no dark adaptation between the pupillary responses to red light during pulse one compared to those during pulse six.

Pulse	Mean ± Standard Deviation	p-value
Mono – Alt Red Pulse 1 minus Pulse 6 under Standard Testing Condition with Dark Adaptation	0.14 ± 0.067	
Mono – Alt Red Pulse 1 minus Pulse 6 under Standard Testing Condition without Dark Adaptation	0.19 ± 0.10	
Pulse 1 minus Pulse 6 Difference due to Dark Adaptation	-0.05 ± 0.11	0.064

Table 6: Paired Differences in Mono minus Alternating Red Light Pupillary Responses During the First and Sixth Pulses of Light Under the Standard Testing Condition with Dark Adaptation and the Standard Testing Condition with No Dark Adaptation

Table 7 shows the effect of spherical equivalent on the outcome measures (single color minus alternating) for the pupillary responses to blue light. Under all five of the conditions, spherical equivalent had no significant correlation to the pupillary responses.

Condition	Correlation with Spherical Equivalent	p-value
Standard 1	-0.37	0.11
Standard 2	-0.25	0.29
Standard No DA	-0.04	0.86
Long Red	-0.34	0.14
Long Red No DA	0.03	0.90

Table 7: Effect of Spherical Equivalent on Mono – Alt Blue Pupillary Response Outcome Measures for the Five Testing Conditions

Tables 8 and 9 show the correlations between the light badge data and pupillary response outcome measures from the same test session. The average light exposure was not significantly different for any testing condition (repeated measures ANOVA, $p = 0.41$). Additionally, as seen in Table 8, the light badge exposure data did not correlate with the pupillary outcomes in response to blue light for any of the testing conditions. In Table 9, one correlation with pupillary outcomes in response to red light was flagged as significant: the correlation between light exposure and pupil outcome in response to red light under the standard testing condition without dark adaptation. Given that the average light exposure was not statistically significantly different between days and that only one correlation between pupillary outcome and light exposure was found, it seems unlikely

that light exposure prior to the pupillary testing condition had any substantial influence on pupillary testing results.

Condition	Average Light Exposure (log lux-minutes)	Correlation with Mono – Alt Blue	P-Value
Standard 1	5.84 ± 0.40	0.248	0.29
Standard 2	5.87 ± 0.61	0.230	0.33
Standard No DA	5.61 ± 0.33	0.192	0.42
Long Red	5.83 ± 0.62	0.169	0.48
Long Red No DA	5.69 ± 0.62	-0.023	0.92

Table 8: Correlations between Light Badge Data and Pupillary Outcomes for Mono-Alt Blue

Condition	Average Light Exposure (log lux-minutes)	Correlation with Mono – Alt Red	P-Value
Standard 1	5.84 ± 0.40	0.290	0.21
Standard 2	5.87 ± 0.61	-0.106	0.66
Standard No DA	5.61 ± 0.33	0.583	0.007*
Long Red	5.83 ± 0.62	0.117	0.62
Long Red No DA	5.69 ± 0.62	-0.019	0.94

Table 9: Correlations between Light Badge Data and Pupillary Outcomes for Mono-Alt Red

Chapter 4: Discussion

The current study found that for the pupillary protocol used, the time spent in dark adaptation does matter. Pupillary response outcomes to blue light as a single color were significantly more constricted under the standard testing condition in which dark adaptation was performed when compared to the standard testing condition in which the participants did not undergo dark adaptation. Additionally, pupillary response outcomes for the testing condition containing dark adaptation with a lengthened amount of red light exposure were significantly different when compared to the standard testing condition under which no dark adaptation was performed. This finding was confirmed in a repeated measures ANOVA adjusting for length of exposure to red as a single color.

The other variable examined in this testing protocol was the effect of lengthening the exposure to single color red light prior to testing pupillary responses to blue light. After adjusting for dark adaptation, this study found that there was no effect of the longer exposure to red light. Ultimately, dark adaptation does produce a significant difference in pupillary responses, but lengthened exposure to red light does not.

Pupillary responses to red light were not significantly different under any of the testing conditions. There was no association between dark adaptation or a lengthened exposure to red light to the pupillary responses elicited by red light. This study also found no

association between light exposure 24 hours prior to pupillary testing and the pupillary response outcomes to either blue or to red.

In addition to finding that time spent during dark adaptation does matter in this pupillary testing protocol, this study also found that the standard pupillary testing protocol is moderately repeatable. The 95% limits of agreement for single color blue minus alternating blue were ±0.078, roughly twice the difference in normalized pupil size of 0.04 between myopic and non-myopic adults (Orr D, Mulvihill S, Shorter P, Hartwick A, Mutti D. The effect of refractive error on red and blue light-driven pupil responses. *Optometry and Vision Science*. 2016;93. Abstract nr. 160093). There were no significant differences between the pupillary response measures for the first trial of the standard testing condition and the second trial of the standard testing condition when looking at the pupillary responses to the flashes of single or alternating blue light. It was important to establish repeatability of this pupillary testing protocol before considering future uses for this method of testing.

Based on the findings from this study, testing with this pupillary protocol should consider including dark adaptation, as dark adaptation was found to make a significant difference in pupillary response measures to blue light. However, it is possible that the important factor was the time spent in dark adaptation rather than darkness itself. In order to make this distinction, another experiment would need to be done to compare the effects of adapting for an equal amount of time in a lit environment compared to in the dark. The hypothesis that the effects seen in this study could be due to time alone is worth

considering. Dopamine has been shown to diffuse through the retina with effects seen in the outer retina in at least five minutes (Bjelke, Goldstein et al., 1996; Sodhi et al., 2014). Although the exact temporal dynamics of retinal diffusion are unknown, five minutes is shorter than the over twenty-minute protocol in the current study, but longer than the two minutes needed for testing red and blue as single colors without dark adaptation.

Whether or not including dark adaptation is important in this testing protocol has practical implications. Ideally, this testing protocol would be utilized in children to assess the differences in pupillary responses for those children who would become myopic versus those children who would emmetropize or develop a hyperopic refractive error. Having a child sit in a dark room for five minutes prior to each round of testing certainly is not an optimal protocol for testing children. However, based on the results of this study, dark adaptation does make a significant difference in the pupillary response outcome measures to blue light, and although not ideal from a practicality standpoint, it should be included in any testing using this pupillary testing protocol.

It is important to note that although the current study found a significant difference in responses to blue light outcomes when comparing conditions with and without dark adaptation, prior studies have been conducted without dark adaptation that still showed a significant difference between myopes and non-myopes when examining a different pupillary outcome measure. A study conducted by Blumenthaler et al. tested children during the summer and the winter, and analyzed the association between axial length and the pupillary responses of children using the standard testing protocol without dark

adaptation. The study found that pupillary constriction in response to blue light compared to red light was greater for children with shorter axial lengths. Interestingly, this association between shorter axial length and smaller pupils in response to blue compared to red was present in summer months but not in winter months (Blumenthaler M, Hartwick A, Mutti DO. The Association Between Axial Length and Pupil Responses to Blue and Red Stimuli in Children Depends on Season. *Optometry and Vision Science*. 2018:95. Abstract nr. 180077).

Both the current study and the previously mentioned study suggest that dark adaptation matters when examining the responses to blue light. Blumenthaler did not find a significant association between axial length and the pupillary outcome measures for single color minus alternating blue light. The outcome measure had to be modified to pupillary responses to blue compared to red, as opposed to blue alone, in order to find the association with axial length. The study conducted by Blumenthaler along with the current study also did not find a relationship between blue light responses and refractive error.

Ultimately when considering the results from both studies, if measuring blue light response alone, dark adaptation matters, and should be included in the testing protocol. However, a more feasible study design for children could be to take out the dark adaptation period and incorporate pupillary responses to red. There was a suggestion in the current study that omitting dark adaptation might modify pupillary responses to red. Comparison of the first and sixth pulses in Figure 16 and 17 suggests that there might be

different pupillary responses to red depending on the presence of dark adaptation. In the current study, this pupillary response analysis did not show a statistically significant difference, although the difference (± SD) was -0.05 ± 0.11, similar to effect sizes related to refractive error, and had a p-value of 0.064.

As briefly mentioned, this study found no association between refractive error and pupillary responses, as would have been expected based on earlier studies using the standard protocol with dark adaptation. This could have been influenced by one highly myopic subject (spherical equivalent = -10.61 D) within the study sample who had robust ipRGC responses. Although the responses from this subject were more typical of data from non-myopes tested in previous studies, eliminating data from that subject did not change any findings. Ultimately, though, it is difficult to pinpoint a definitive explanation for why no such association was found. Variation in the causes of myopia development might play a role. Refractive error represents a combination of factors including genetics, the amount of light exposure from time outdoors, when that exposure occurred recently and in the past, possibly as far back as childhood, and perhaps how ipRGCs respond to light exposure in terms of the release of dopamine or other modulators of eye growth.

Not finding an association between refractive error and pupillary responses could point to a weakness in the study. The study had primarily myopic subjects (15/20), meaning that emmetropes and hyperopes were under-represented in the study. Including subjects with a wider range of refractive errors might have increased the likelihood of finding an association between pupillary responses and refractive error.

There were also several strengths to this study. One such strength was that the sample size was based on planning and calculations that were conducted prior to the study. Participant recruitment was successful in obtaining an adequate number of participants based on these calculations. Additionally, participant retention was a strength of this study, as none of the participants dropped out from the study after beginning testing. All subjects were between the ages of 18 and 30, removing age as a significant confounding variable. Of the 20 participants, 11 were female and 9 were male, so each gender was well represented in this study. Another strength involves the collection of light badge data. Light badge data was collected and analyzed for each participant to ensure that light exposure was not a confounding variable in pupillary response outcome measures (Abbott et al., 2018). Additionally, the study took place in the summertime, so light exposure was more intense and participants were unlikely to obstruct light data collection with long sleeves or jackets.

Although the collection and analysis of light badge data was certainly a strength in this study, one weakness in the collection of this data could be the length of time for which light badge data was analyzed prior to pupillary testing. In this study, only 24 hours prior to testing was considered in analysis of light badge data. It perhaps would have been advantageous to analyze a longer time period of light exposure prior to testing.

Another strength of the study was the randomization in which participants underwent the various pupillary testing protocols. Each participant underwent the five testing conditions

in a randomized order, ensuring there was no effect of the order in which participants underwent each testing condition.

There are many future studies that could be conducted in order to delve further into the association of ipRGC responses with refractive error utilizing the pupillary testing protocol. It would be interesting to conduct a longitudinal study in children, analyzing pupillary responses and comparing these to the development of refractive error. Although the current study showed that light badge data did not make a difference in pupillary response measures, it would also be interesting to collect this data in children over a longer period of time to determine the effect of light exposure on the development of refractive error and pupillary testing outcome measures. This study provides evidence to answer the original question of whether or not the pupillary testing protocol produces repeatable pupillary responses. The evidence found supports the idea that the protocol is moderately repeatable, and that degree of repeatability can be incorporated into the planning of future studies. The outcomes found in this study also suggest that dark adaptation is a critical component of the pupillary testing protocol when analyzing the pupillary responses to blue light, but that adding a lengthened amount of exposure to red light prior to the testing sequence does not make a significant difference.

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